Role of a Pertussis Toxin–Sensitive Protein in the Modulation of Canine Purkinje Fiber Automaticity


We previously have shown that α-adrenergic stimulation of canine Purkinje fibers and rat ventricle decreases automaticity. Experiments on rat ventricular myocytes in tissue culture have suggested that the decrease in automaticity induced by α-adrenergic stimulation depends on the development of sympathetic innervation and the presence of a pertussis toxin–sensitive, 41-kDa guanosine triphosphate (GTP)–regulatory protein. In the present study, microelectrode and biochemical techniques were used to test the role of the pertussis toxin–sensitive protein and sympathetic innervation in modulating automaticity of adult canine Purkinje fibers. Fibers were incubated in Tyrode’s solution alone or in Tyrode’s solution plus pertussis toxin (0.1–0.5 μg/ml) for 24 hours and were then superfused with phenylephrine. Phenylephrine in the $5 \times 10^{-5}$–$5 \times 10^{-4}$ M range induced a decrease in automaticity in 63% of the fibers not treated with pertussis toxin and an increase in automaticity in 37%. The former group had a higher level of pertussis toxin–sensitive substrate by the [3H]nicotinamide adenine dinucleotide adenosine diphosphate (ADP)–ribosylation assay than the latter. In contrast, all fibers treated with pertussis toxin (0.5 μg/ml) showed increased automaticity in response to phenylephrine and had no detectable pertussis toxin–sensitive substrate. Over the range of pertussis toxin concentrations studied, there was a smooth concentration-response relation between the substrate levels measured and the automatic response to phenylephrine. As ADP-ribosylatable substrate levels decreased, the percent of fibers showing an increase in automaticity increased. In fibers from dogs postsympathectomy with 6-hydroxydopamine, the percent showing increased or decreased automaticity of adult canine Purkinje fibers. Fibers were incubated in Tyrode’s solution alone or in Tyrode’s solution plus pertussis toxin (0.5 μg/ml) and had no detectable pertussis toxin–sensitive substrate. Moreover, we hypothesized that in the remaining adult fibers showing a positive chronotropic response to α-adrenergic stimulation, this response is dependent on the presence of an N-protein that is a pertussis toxin–sensitive substrate.5

The data to be presented will demonstrate that fibers showing a negative chronotropic response to phenylephrine are enriched in an N-protein that is a pertussis toxin–sensitive substrate and that presumably serves as a transducer of this negative chronotropic response.
Moreover, after exposure of these fibers to pertussis toxin, which adenosine diphosphate (ADP)-ribosylates and functionally inactivates the pertussis toxin-sensitive substrate, the response to α-adrenergic stimulation reverts to positive chronotropy.

Materials and Methods

The general approach is shown schematically in Figure 1. Pentobarbital (30 mg/kg i.v.) was used to anesthetize healthy adult mongrel dogs of either sex weighing 15–30 kg. The heart was removed through a right lateral thoracotomy, and Purkinje fiber bundles were excised from the right and left ventricles. The bundles were placed in cold Tyrode’s solution and gassed with 95% O₂-5% CO₂ at pH 7.3. The composition of the Tyrode’s solution was (in mM) NaCl 131, NaHCO₃ 18, NaH₂PO₄ 1.8, KCl 2.7, CaCl₂ 2.7, MgCl₂ 0.5, and dextrose 5.5.

For those studies in which sympathetic denervation was required, the methods of Alter et al.⁶ and Burks et al.⁷ were used to administer 6-hydroxydopamine. A total of 50 mg/kg was injected intravenously over 6 days in doses as follows: 1, 3, 5, 10, 11, and 20 mg/kg. After the dogs had stabilized for 1 week, the Purkinje fibers were removed as described above.

In a separate series of experiments, the catecholamine contents of normal and chemically sympathectomized Purkinje fibers were measured using a radioenzymatic assay technique.⁸ To further estimate the degree of denervation established by 6-hydroxydopamine, the effects of tyramine (10⁻⁵ M) on the spontaneous rate of Purkinje fibers from neurally intact and sympathectomized dogs were determined.

For cellular electrophysiological studies related to the effects of exposure to pertussis toxin, the ADP-ribosylation of the GTP-regulatory protein by the toxin had to be ensured; this required a 24-hour incubation period. To be consistent among the experimental protocols, the 24-hour incubation period was used for both treated and untreated Purkinje fibers. For this reason, all fibers were processed from neurally intact and 6-hydroxydopamine-treated dogs as follows: After excision from the heart and removal of excess muscle, groups of three fiber bundles were placed in beakers containing 100 ml Tyrode’s solution. The final concentration of pertussis toxin in the beakers was 0.1–0.5 μg/ml. All beakers were covered with Parafilm, and the contents were gassed continuously with 95% O₂-5% CO₂; then the beakers stood for 24–29 hours at room temperature. This procedure had no apparent deleterious effects on the electrophysiological properties or the automaticity of the Purkinje fibers (see below).

Fibers from at least two animals were studied during each experiment, and the protocols among dogs and fiber types (i.e., right bundle branch system and anterior and posterior divisions of the left bundle) were randomized so that fibers from each animal were used for the two protocols (pertussis toxin treatment or control) and fibers from both ventricles were distributed among all interventions.

Microelectrode Techniques

The fibers were placed in a tissue bath perfused with Tyrode’s solution that was gassed as described above. The perfusate was maintained at 37.3 ± 0.2°C and the flow rate at 12 ml/min, which ensured an exchange of bath contents three times per minute. The tissues beat spontaneously and were impaled with 3 M KCl-filled glass capillary microelectrodes with resistances of 15–25 MΩ. The bath was connected to ground via a 3 M KCl-Ag-AgCl bridge, and an identical bridge was used to couple the electrodes to amplifiers having high input impedance and capacity neutralization. The calibration of this system has been described in detail previously.² The signals were displayed on oscilloscopes and on a strip chart recorder, and previously described methods⁹ were used to measure maximum diastolic potential, activation voltage, overshoot of phase 0, and automatic rate.

Experimental Protocols

The fibers equilibrated in the tissue bath for 30–45 minutes, after which a regular automatic rhythm (with variability <10%) was usually demonstrable. If the rhythm was too irregular to permit accurate measurement, the fibers were discarded.

![Figure 1. Experimental protocol. (See text for description.)](http://circres.ahajournals.org/Download)
Then the following experiments were performed on normal fibers and on those from 6-hydroxydopamine-treated dogs: Following equilibration in the tissue bath, fibers were superfused with phenylephrine in graded concentrations of $5 \times 10^{-6}$ to $5 \times 10^{-5}$ M. Each superfusion period was 12 minutes long (in preliminary studies, phenylephrine was found to attain a steady-state effect on rate within 5 minutes). Following control and at the end of each superfusion period, the transmembrane potential characteristics described above were measured. Also, the automatic rate for the last 4 minutes of each superfusion period was measured to quantify the effect of phenylephrine on automaticity. This protocol was used for the pertussis toxin-treated fibers as well. It is to be emphasized that over the concentration range of phenylephrine ($5 \times 10^{-6}$ to $5 \times 10^{-5}$ M), all fibers eventually show a positive chronotropic response. However, the effect at agonist concentrations $\geq 10^{-5}$ M is largely $\beta$-adrenergic. For the purpose of the present study, we were interested solely in the $\alpha$-adrenergic effect, which is an increase or decrease in automaticity usually seen in the $10^{-6}$ to $5 \times 10^{-5}$ M concentration range. Hence, throughout the present study, when we refer to a phenylephrine-induced decrease or increase in automaticity, we are referring to one that is $\alpha$-adrenergic and occurs over the low phenylephrine concentration range.

Further experiments were performed on those fibers that showed a positive chronotropic response to $5 \times 10^{-5}$ and/or $5 \times 10^{-4}$ M phenylephrine (see “Results”) to verify whether this response resulted from an $\alpha$- or $\beta$-adrenergic effect. In these experiments, the $\alpha$-blocker prazosin ($10^{-4}$ to $10^{-6}$ M) was used. This range of concentrations has previously been shown to have no significant effect on the Purkinje fiber transmembrane potential or automaticity. The $\beta$-blocker used was propranolol ($2 \times 10^{-7}$ M), a concentration that has no significant effect on the Purkinje fiber transmembrane potential or automaticity.

**Biochemical Techniques**

After the electrophysiological experiments described above were performed, the same Purkinje fibers were evaluated for the presence of a substrate for pertussis toxin–catalyzed ADP-ribosylation. Immediately on completion of the microelectrode studies, membranes from groups of Purkinje fibers representative of each experimental condition were prepared. Each group consisted of 2–5 fibers having wet weights of 6–13 mg/fiber. The pooled Purkinje fibers were suspended in 1 ml sucrose ($0.25$ M)-Trizma ($0.05$ M) buffer (pH 7.6), homogenized at $4^\circ$ C with a Teflon-coated pestle (5,400 rpm x 1 minute), and centrifuged at 43,600g for 45 minutes. The soft pellet was resuspended in an appropriate volume of sucrose-Trizma buffer (approximately 200 $\mu$L) to achieve a final protein concentration of approximately 0.8 mg/ml. ADP-ribosylation assays were performed according to the method of Kaslow et al with modifications. Pertussis toxin was activated by incubation with 10 mM dithiothreitol for 10 minutes at $30^\circ$ C, and 25 $\mu$L Purkinje fiber membrane (20 $\mu$L) was incubated in 65 $\mu$L of a $50$ mM potassium phosphate buffer (pH 7.5) containing 10 U aprotinin, 0.2 mM GTP, 13 mM thymidine, 3.2 mM ADP-ribose, 13 mM arginine, and 10 $\mu$M [$^{32}$P]nicotinamide adenine dinucleotide (NAD) (18–54 ci/mmole) in the presence or absence of pertussis toxin (2 $\mu$g) for 20 minutes at $30^\circ$ C. The reaction was terminated by adding 1 ml ice-cold 7% trichloroacetic acid (TCA) and centrifuging at 12,000g. The pellet was washed by resuspension in 1% TCA and recentrifugation at 12,000g and then was solubilized with sodium dodecyl sulfate (SDS) sample buffer (1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl [pH 6.8], and 0.02% bromophenol blue). Electrophoresis was performed on vertical slab gels (resolving gel 10%, stacking gel 5% acrylamide) at 300 V for 3 hours; the gels were stained with Coomassie blue dye and autoradiographed by exposure to Kodak XRP-5 film. The specific protein band on the polyacrylamide gel that was coincident with the labeled band on the autoradiogram was removed and counted using liquid scintillation spectroscopy. The concentration of pertussis toxin–sensitive protein was determined by relating the number of counts in this band of interest to the specific activity of [$^{32}$P]NAD and to the protein concentration used.

**Data Analysis**

Transmembrane potentials are reported only from single impalements maintained throughout the experimental protocol. The single fiber bundle was the unit of analysis in all experiments. The statistical technique used was analysis of variance, with Scheffe's test when the $F$ value (ANOVA) permitted this. In instances where concentration-response curves were compared, a nested ANOVA was used. Results are expressed as the mean ± SEM.

**Pharmacological Agents**

We purchased phenylephrine, tyramine, and propranolol from Sigma Chemical Co., St. Louis, Missouri, and pertussis toxin from List, Campbell, California. Prazosin was a generous gift from Pfizer, Groton, Connecticut.

**Results**

The control transmembrane potential characteristics of 16 Purkinje fibers are presented in Table 1. The fibers were grouped based on their response to phenylephrine ($5 \times 10^{-6}$ and $5 \times 10^{-5}$ M) subsequent to the control measurement. Ten of the fibers (63%) showed a negative chronotropic response to phenylephrine ($5 \times 10^{-6}$ or $5 \times 10^{-5}$ M); the remainder showed a positive response. The phenylephrine dose–response curves for the two groups of fibers are presented in Figure 2. It is apparent from this figure that $5 \times 10^{-5}$ M phenylephrine reduced automaticity significantly in 10 fibers and increased automaticity in the remaining six fibers. As stated above, the increase in automaticity occurring in both populations of fibers at higher agonist concentrations is $\beta$-adrenergic. The amount of detect-
Note lesser density of 41-kDa band in latter group. (See text for previously been shown to be a $\beta$-adrenergic effect. *p<0.05 of phenylephrine. Response is graphed as change in rate; control fibers showing decreased automaticity (23 fmol/mg) and one group having a negative (⋄) chronotropic response.

This observation led us to ask two questions: First, was the presence of pertussis toxin–sensitive substrate related to the type of electrophysiological response that occurred? Second, what was the effect of sympathetic denervation on the response to $\alpha$-adrenergic stimulation and the detectable pertussis toxin–sensitive substrate? We derived the second question, in part, from our previous observation that in cultured rat myocytes, the development of sympathetic innervation is associated with increased levels of pertussis toxin substrate and conversion of the effect of $\alpha$-adrenergic stimulation from positive to negative chronotropy.4-5 To answer the first question, a group of adult fibers were incubated with pertussis toxin. We reasoned that if the pertussis toxin–sensitive substrate is important in modulating the chronotropic response in adult fibers, its functional inactivation (i.e., by exposure to pertussis toxin) should cause reversion of the response to $\alpha$-adrenergic stimulation from inhibition to excitation. To answer the second question, chemical sympathectomy was performed on another group of dogs.

Table 2 includes the control transmembrane potentials for 25 fibers treated with pertussis toxin (0.1–0.5 $\mu$g/ml) for 24 hours prior to electrophysiological study. Comparison of transmembrane potentials within each group and with fibers receiving no pertussis toxin (Table 2) reveals no consistent change in any parameter measured in the absence or presence of any concentration of pertussis toxin (0.1–0.5 $\mu$g/ml, p>0.05). For example, the range of automatic rates for all groups of fibers showing a negative chronotropic response was 9–28 beats/min; that for groups showing a positive response was 9–35 beats/min (p>0.05 for the two groups). Irrespective of exposure to pertussis toxin and of the presence of a positive or negative chronotropic response, concentrations of $<5 \times 10^{-7}$ M phenylephrine had no significant effect on maximum diastolic potential, activation voltage, or overshoot (data not shown).

Table 2. Transmembrane Potential Characteristics and Automaticity of Purkinje Fibers Incubated in Tyrode's Solution With Pertussis Toxin for 24 Hours

<table>
<thead>
<tr>
<th>Pertussis toxin (µg/ml)</th>
<th>Maximum diastolic potential (mV)</th>
<th>Activation voltage (mV)</th>
<th>Overshoot (mV)</th>
<th>Rate (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=2)</td>
<td>96</td>
<td>93</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>88 ± 3.4</td>
<td>84 ± 2.0</td>
<td>30 ± 0.3</td>
<td>17 ± 7.0</td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>94 ± 5.3</td>
<td>91 ± 4.9</td>
<td>33 ± 2.9</td>
<td>21 ± 8.4</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=3)</td>
<td>103 ± 5.5</td>
<td>95 ± 11.0</td>
<td>33 ± 2.9</td>
<td>28 ± 12.2</td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=4)</td>
<td>95 ± 2.9</td>
<td>86 ± 2.3</td>
<td>26 ± 3.7</td>
<td>16 ± 4.8</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=2)</td>
<td>93</td>
<td>88</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>94 ± 4.1</td>
<td>87 ± 5.3</td>
<td>32 ± 5.8</td>
<td>17 ± 4.0</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Group 1 includes those fibers that subsequently showed a positive chronotropic response to phenylephrine ($5 \times 10^{-9}$ and/or $5 \times 10^{-8}$ M); group 2 includes those fibers that subsequently showed a negative chronotropic response to phenylephrine ($5 \times 10^{-9}$ and/or $5 \times 10^{-8}$ M).

SEM calculated only when n=3.
A representative experiment depicting the effects of phenylephrine on one fiber that was and another that was not incubated in pertussis toxin is presented in Figure 3. Note the increased automaticity at $5 \times 10^{-9}$ M phenylephrine in the former and the decreased automaticity in the latter.

The relation between detectable pertussis toxin-sensitive substrate and phenylephrine-induced changes in automaticity was examined further by comparing these two features under control conditions and at the different concentrations of pertussis toxin studied. We reasoned that if the ability of pertussis toxin to functionally inactivate (i.e., ADP-ribosylate) the regulatory protein determines whether there will be a negative chronotropic response, a consistent relation between substrate availability and phenylephrine-induced changes in automaticity in a given group of fibers should be seen. Therefore, the relation between the concentration of pertussis toxin and the chronotropic response to phenylephrine was tested first. Phenylephrine increased automaticity in 37% of 16 fibers that had not been exposed to pertussis toxin and decreased it in the remaining 63%. At 0.1 µg/ml pertussis toxin, approximately the same result was seen as in the absence of toxin: 33% of six fibers showed an increase and 67% showed a decrease in automaticity. However, at a higher toxin concentration (0.25 µg/ml), a greater proportion of fibers (57% of seven fibers) showed an increase in automaticity; at 0.3 µg/ml, 67% of six fibers showed an increase; and at the highest concentration (0.5 µg/ml), 100% of six fibers showed an increase. Hence, a concentration-response relation between the pertussis toxin concentration and the effect of phenylephrine on automaticity was demonstrated.

In Figure 4, the pertussis toxin substrate in control and at concentrations of 0.25 and 0.5 µg/ml has been quantified and related to the percent of fibers that showed a positive or negative chronotropic response to phenylephrine. It is apparent that under control conditions, detectable pertussis toxin-sensitive substrate is higher for the group that showed a negative chronotropic response than for the group that showed a positive response. At 0.5 µg/ml pertussis toxin, no substrate is demonstrable, and all fibers show increased automaticity. At 0.25 µg/ml toxin, the percent of fibers showing a negative chronotropic response is reduced, and the substrate levels for both groups of fibers are shown). At the higher phenylephrine concentrations, a variable (2–4 mV) increase in maximum diastolic potential and activation voltage occurred that attained significance ($p<0.05$) only for the group that received no pertussis toxin (data not shown).
by guest on April 30, 2017 http://circres.ahajournals.org/ Downloaded from

fibers than it was in absence of toxin (n = one group of fibers for =
protein concentration was 30.9 fmol/mg for fibers showing
of sympathetic denervation to the available N-protein
with 6-hydroxydopamine. The Purkinje fiber cate-
and the a-adrenergic response. Six dogs were injected
for control electrophysiologic data on fibers and
detectable pertussis toxin-sensitive substrate (upper panel) and detectable pertussis toxin-sensitive substrate
(lower panel) to pertussis toxin concentration (horizontal axis). For fractional response to phenylephrine, control = 0, and + 1 and − 1 represent 100% of fibers showing increase or decrease in automaticity, respectively. For substrate levels, values presented are means for pooled fibers. In absence of toxin, approximately two thirds of fibers show decrease in automaticity, and detectable level of substrate is higher for fibers showing negative rather than positive chronotropic response (n = three groups of fibers for former, two for latter). At toxin concentration of 0.25 μg/ml, 57% of fibers now show increase in automaticity. Detectable substrate is lower in both groups of fibers than it was in absence of toxin (n = one group of fibers for each response). At toxin concentration of 0.5 μg/ml, all fibers show increase in automaticity, and no pertussis toxin-sensitive substrate is demonstrable (n = two groups). Data for pertussis toxin (0.1 and 0.3 μg/ml) are not included. At 0.1 μg/ml, N protein concentration was 30.9 fmol/mg for fibers showing decreased automaticity (n = one group) and 5.9 fmol/mg for those showing increased automaticity (n = one group). At 0.3 μg/ml toxin, N protein concentration was 0.32 fmol/mg for fibers showing decreased automaticity (n = one group) and was undetectable for those showing increased automaticity (n = one group). For control electrophysiologic data on fibers and number of fibers in each group, seeTables 1 and 2.

lower than the controls. These results indicate that in
fibers having a negative chronotropic response, the available pertussis toxin substrate concentrations were
always higher than in those having the positive
response. Moreover, even in the presence of pertussis
toxin, which reduces the detectable substrate in a
concentration-dependent fashion, the pertussis toxin
substrate levels remained higher in those fibers showing
a negative response.

This series of experiments indicated that a
concentration-response relation could be demonstrated
between the level of pertussis toxin substrate and the
and detectable pertussis toxin-sensitive substrate levels remained higher in those fibers showing a
negative response.

This series of experiments indicated that a
concentration-response relation could be demonstrated
between the level of pertussis toxin substrate and the
type of response to phenylephrine. We, therefore,
turned to our second question concerning the relation
of sympathetic denervation to the available N-protein
and the α-adrenergic response. Six dogs were injected
with 6-hydroxydopamine. The Purkinje fiber cate-
cholamine (epinephrine and norepinephrine) levels of
these dogs were 18 ± 6 ng/g protein compared with
611 ± 153 ng/g protein for eight dogs that had not been
sympathectomized. The cellular electrophysiology of
11 Purkinje fibers from the 6-hydroxydopamine-
treated dogs were studied. The control automatic rate
of these fibers was 14 ± 2.0 beats/min. Following
exposure to 10⁻⁵ M tyramine, the rate was 17 ± 4.6
beats/min (p>0.05). In contrast, the control automatic
rate for six Purkinje fibers from four dogs that did not
receive 6-hydroxydopamine was 11 ± 2.9 beats/min.
Following superfusion with 10⁻⁴ M tyramine, the rate
increased to 31 ± 3.7 beats/min (p<0.01). Hence, both
biochemically and physiologically, there was evidence
of denervation and catecholamine depletion secondary
to administration of 6-hydroxydopamine.

Of the 11 fibers from 6-hydroxydopamine–treated
dogs, eight showed a negative and three showed a
positive chronotropic response to phenylephrine
(5 × 10⁻⁹–5 × 10⁻⁸ M) (Figure 6), a proportion that is
comparable to data observed for innervated dogs. The
level of pertussis toxin substrate for the group showing
depression in automaticity was 11.7 fmol/mg protein
(mean of two groups of pooled fibers); no substrate was
detectable in the fibers having a positive chronotropic
response. Although the value of 11.7 fmol/mg protein
in the negative chronotropic group is lower than that
shown in Figure 5 for negative or positive responders,
in the group of nine fibers from dogs that were not
treated with 6-hydroxydopamine but served as con-
current controls, the pertussis toxin substrate for the six
fibers showing a negative chronotropic response was
11.0 fmol/mg (mean of two groups of pooled fibers).
Hence, 6-hydroxydopamine induced no reduction in
substrate, as seen in the comparable concurrent control.

The control transmembrane potentials for the groups
showing negative and positive chronotropy were,
respectively, maximum diastolic potential, −95 ± 1.5
and $-96 \pm 1.2$ mV; activation voltage, $-84 \pm 2.2$ and $-82 \pm 6.6$ mV; and overshoot, $37 \pm 1.9$ and $25 \pm 9.4$ mV. No significant response of these variables to phenylephrine $\leq 5 \times 10^{-7}$ M was found. At higher concentrations, the fibers having a negative chronotropic response showed a significant increase in maximum diastolic potential (5 mV, $p<0.05$).

These results indicated to us that during the 2-week interval following the onset of 6-hydroxydopamine treatment, there was no change in the detectable pertussis toxin–sensitive substrate or in the $\alpha$-adrenergic response. At this point, we considered an additional question: Given that phenylephrine has both $\alpha$- and $\beta$-adrenergic properties, could we be confident that there was an $\alpha$-adrenergic contribution to the increase in automaticity at low phenylephrine concentrations?

To answer this question, another group of fibers were incubated in pertussis toxin (0.5 $\mu$g/ml) for 24 hours. Whether the positive chronotropic response elicited by phenylephrine ($5 \times 10^{-8}$ M) could be blocked by propranolol or by prazosin was tested first. For the group exposed to propranolol ($2 \times 10^{-7}$ M), the control maximum diastolic potential was $-95 \pm 2.9$ mV, activation voltage was $-85 \pm 3.5$ mV, and overshoot was $19 \pm 5.8$ mV. As shown in Figure 7, these fibers developed a positive chronotropic response to phenylephrine ($5 \times 10^{-8}$ M) despite the presence of propranolol. This was blocked by $10^{-6}$ M prazosin.

Experiments were performed using prazosin but not propranolol on five additional fibers that also had been preincubated in pertussis toxin (0.5 $\mu$g/ml). The control transmembrane potentials were maximum diastolic potential, $-93 \pm 2.2$ mV; activation voltage, $-85 \pm 3.5$ mV; and overshoot, $36 \pm 4.2$ mV. Phenylephrine ($5 \times 10^{-8}$ M) significantly increased automaticity in these fibers (Figure 8A). This response was reduced in a concentration-dependent manner by adding prazosin ($10^{-6} \text{ to } 10^{-4}$ M) to the superfusate. Subsequent washout of the prazosin resulted in a partial return toward the automatic rate seen in phenylephrine alone. In another group of 7 fibers that had not been exposed to pertussis toxin, the effects of prazosin on the positive chronotropic response elicited by phenylephrine ($5 \times 10^{-8}$ M) were tested. Here, the control transmembrane potentials were maximum diastolic potential, $-94 \pm 3.3$ mV; activation voltage, $-86 \pm 3.6$ mV; and overshoot, $21 \pm 2.7$ mV. As shown in Figure 8B, $10^{-6}$ M prazosin completely abolished the positive chronotropic response.

Discussion

In summary, superfusion of adult canine Purkinje fibers with low concentrations of phenylephrine has two effects on automaticity: An increase is seen in approximately one third and a decrease is seen in the remainder. For those fibers in which there is a decrease in automaticity, the levels of a pertussis toxin–sensitive substrate are approximately twice those seen for the population in which automaticity increases. When the substrate is ADP-ribosylated and thereby inactivated by pertussis toxin, there is a progressive decrease in the population of fibers that shows an $\alpha$-adrenergic–induced decrease in automaticity. At the highest concentration of toxin used, the substrate is not measurable and automaticity uniformly increases.

It is quite clear that following exposure to high pertussis toxin concentrations, where the substrate is totally inactivated, the increase in automaticity is an $\alpha$-adrenergic phenomenon. We state this because the response to phenylephrine is blocked completely by prazosin, in a concentration-dependent fashion, and is unaltered by a $\beta$-blocking concentration of propranolol. Based on this information, it is reasonable to propose a role for the pertussis toxin–sensitive substrate in coupling the $\alpha$-receptor to its effector. We assume that when pertussis toxin–sensitive substrate is demonstrable, coupling is to a process that inhibits automaticity, and that when substrate is functionally inactivated, coupling is to a process that excites automaticity.

Although the nature of the effector systems to which pertussis toxin is linked is not yet known, investigation of the relation between the pertussis toxin–sensitive substrate and pacemaker currents is ongoing. Results to date suggest the decrease in automaticity induced by $\alpha$-adrenergic stimulation is the result of stimulation of Na$^+$-K$^+$ pump current by the pertussis toxin–sensitive substrate.$^{19}$ ADP-ribosylation of the substrate with pertussis toxin abolishes the $\alpha$-adrenergic–induced stimulation of pump current. This same study also has shown a pertussis toxin–sensitive substrate–linked decrease in gK.$^{14}$ Such an event would tend to increase automaticity. Hence, it must be assumed that the decrease in automaticity seen in fibers treated with $\alpha$-agonists reflects a predominant effect on the pump current.
Although investigation of this aspect of the modulation of automaticity is still preliminary, other features of α-adrenergic modulation of automaticity can be discussed with greater confidence. We and others already have determined that α-adrenergic inhibition of automaticity is seen mainly in well-polarized fibers of the canine ventricular conduction system and human atrium. As in the present study, our earlier experiments were performed largely on fibers having maximum diastolic potentials negative to −85 mV. However, Purkinje fibers depolarized via current injection to low levels of membrane potential (<−60 mV) as well as sinus node electrophysiology have been studied in isolated rabbit and intact canine hearts. In all three situations, α-adrenergic stimulation does not decrease automaticity; there is, if anything, a tendency towards a slight increase. That α-adrenergic stimulation may increase automaticity was also demonstrated independently in a study of Ba²⁺-depolarized Purkinje fibers. Hence, it is likely that the pertussis toxin–sensitive substrate modulates the effect of α-adrenergic stimulation on automaticity at high membrane potentials, but when pacemaker fibers are depolarized to low potentials—even in the presumed presence of substrate (i.e., in Purkinje fibers subjected to depolarizing current)—the modulation of pacemaker activity is lost. The failure to see an inhibitory effect of α-adrenergic stimulation on automaticity in fibers having a low membrane potential (presumably modulated by the i₅ current) may indicate that there is no linkage of the substrate to this pacemaker mechanism. However, the observations by Shah and Cohen of a substrate-dependent decrease in gK also would provide a means by which the α-adrenergic–induced increase in automaticity might occur in depolarized fibers.

Although the present study has shown that the increase in automaticity induced by low concentrations of phenylephrine is inhibited by α-adrenergic blockade, it has been previously demonstrated that comparable increases in automaticity in adult canine fibers were blocked by the β-blocker propranolol. We believe this apparent contradiction may be explained by the following: First, in the earlier study, Purkinje fibers were used immediately after their excision from the heart. The present study used Purkinje fibers 24 hours after excision. Second, in the earlier study, there was a wide variance in the response to the β-blocker, and no α-blocker was studied. Third, in the present protocol, phenylephrine was added after propranolol had reached a steady state. It is possible, indeed likely, that a partial block of the phenylephrine effect would have been elicited by propranolol had we designed the protocol to investigate β-blockade. Hence, differences in experimental protocol are likely to explain the disparate results of the two studies.

As stated earlier, studies of neonatal dogs and rat myocytes in tissue culture and coculture with sympathetic neurons have led us to suggest that sympathetic innervation is a key factor in the induction of an increase in tissue levels of the pertussis toxin–sensitive

![Figure 8](http://circres.ahajournals.org/)

**FIGURE 8.** Panel A: Response to phenylephrine and prazosin of five fibers exposed to pertussis toxin (0.5 μg/ml). Control rate, 21 ± 6.3 beats/min. After 24 hours of exposure to toxin, fibers were superfused with phenylephrine (5 × 10⁻⁴ M). Phenylephrine was continued and three concentrations of prazosin were added, followed by a wash in phenylephrine, alone for 30 minutes, y axis, change in rate (beats/min). Panel B: Effects of prazosin (10⁻⁵ M) on increase in automaticity induced by phenylephrine in seven fibers not exposed to pertussis toxin. y axis, change in rate (beats/min); control, 29 ± 6.6 beats/min; x axis, control followed by superfusion with phenylephrine (5 × 10⁻² and 5 × 10⁻⁴ M). Phenylephrine was then washed out and fibers were superfused with prazosin (10⁻⁵ M) for the duration of the experiment. Reexposure to phenylephrine resulted in no further change in rate. *p<0.05 of control.
substrate. Our results with 6-hydroxydopamine suggest that once a certain tissue level of substrate is attained, sympathetic innervation need not be maintained to sustain the α-adrenergic decrease in automaticity. Rather, as shown in Figure 6, both the magnitude of the decrease in automaticity and the proportion of fibers demonstrating it are comparable to those in control situations. What remains unanswered in these studies is whether there is a decline in demonstrable substrate levels over a longer period than the brief interval considered so that ultimately, excitation of automaticity would be seen. The answer to this question would have important implications regarding rhythms following cardiac transplantation as well as in the setting of myocardial infarction and also would provide a definitive response to whether sustained innervation is required to maintain tissue levels of the pertussis toxin–sensitive substrate.

A final consideration here relates to the identity of the substrate for pertussis toxin described in these studies. Several GTP-binding proteins of similar molecular weights (39–41 kDa) have been identified as substrates for pertussis toxin, including NTR, Nt, transducin, and a recently described neutrophil N-protein.2021 In our studies, total ADP-ribosylatable pertussis toxin substrate was measured. A single labeled band in the molecular weight range of approximately 41 kDa was always observed. However, the similar mobility of virtually all pertussis toxin substrates by SDS-PAGE makes it difficult to separate one from another using this technique. It is distinctly possible that Purkinje fibers contain more than one species of pertussis toxin substrate and that only one of these proteins specifically couples the α-adrenergic receptor to the negative chronotropic response. Immunologic techniques that discriminate among the different species of N-proteins will be useful to identify the N-proteins present in Purkinje fibers and to implicate rigorously which specific N-protein is involved in the α-adrenergic negative chronotropic response.

Acknowledgments

The authors express their gratitude to Drs. Irina Golyakhovsky and John Mill for their assistance in performing some of the experiments, to Sharon Miller for her technical assistance, and to Linda Morris and Susan McMahon for their careful attention to the preparation of the manuscript.

References


Key Words: α-adrenergic stimulation • GTP regulatory proteins • cardiac-autonomic interactions
Role of a pertussis toxin-sensitive protein in the modulation of canine Purkinje fiber automaticity.
M R Rosen, S F Steinberg, Y K Chow, J P Bilezikian and P Danilo, Jr

doi: 10.1161/01.RES.62.2.315

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/62/2/315